PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
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in its capacity as elected Office

Date of mailing (day/month/year) 04 October 2000 (04.10.00)	
International application No. PCT/SE00/00073	**Applicant's or agent's file reference** 2006267
International filing date (day/month/year) 14 January 2000 (14.01.00)	**Priority date** (day/month/year) 15 January 1999 (15.01.99)
Applicant ERIKSSON, Peter et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

12 July 2000 (12.07.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2006267		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/SE00/00073	14.01.2000	15.01.1999
International Patent Classification (IPC) or national classification and IPC7		
C12N 15/64, A61K 48/00, C12Q 1/68		
Applicant		
A+ Science Invest AB et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12.07.2000	12.04.2001
Name and mailing address of the IPEA/SE	Authorized officer
Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. 08-667 72 88	Telex 17978 PATOREG-S Yvonne Siösteen/BS Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1998)

INTERNATIONAL PRE[redacted]NARY EXAMINATION REPORT

[redacted]ational application No.
PCT/SE00/00073

I. Basis of the report

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed

☐ the description:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the claims:
pages _____, as originally filed
pages _____, as amended (together with any statement) under article 19
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the drawings:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the sequence listing part of the description:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☒ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheet/fig _____

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2 (c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
[redacted]/SE00/00073

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 24-35 (partially), 37 (partially), 39-40, 43, 45

because:

☒ the said international application, or the said claims Nos. 24-35 (partially), 37 (partially), 39-40, 43, 45 relate to the following subject matter which does not require an international preliminary examination *(specify)*:

See PCT Rule 67.1 (iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _____ are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for said claims Nos. _____

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRE[redacted]ARY EXAMINATION REPORT

[redacted]national application No.
PCT/SE00/00073

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-38,41-42,44	YES
	Claims		NO
Inventive step (IS)	Claims	1-38,41-42,44	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-38,41-42,44	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

The present invention relates to a method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell. In the method the nucleic acid directly interacts with the cell membrane or a component within said cell membrane. Subsequently, the substance comprising said nucleic acid is taken up by the cell via an inherent transport mechanism of the cell. The internalisation mechanism employed is unique to neural stem cells and progenitor cells. The method can for example be used in identification of stem cells or progenitor cells and in gene therapy.

The International Search revealed six documents indicating the state of the art:

A. WO96/15811 A1
B. WO94/25608 A1
C. Boado RJ et al. Journal of Pharmaceutical Sciences, 87 (1998), 1308-1315
D. Kato Y et al. Critical Reviews in the Therapeutic Carrier Systems, 14 (1997), 287-331
E. US5750376
F. WO97/44442A1

Claims 24-35 and 37 may refer to in vivo medical treatment, and are not required to be examined in this aspect. The judgement below refers to methods that are industrially applicable.

None of the documents revealed in the International Search disclose the internalisation mechanism used in the method of the present application. Claims 1-38, 41-42 and 44 are considered to fulfil the requirements of novelty, inventive step and industrial applicability.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD FOR INTRODUCING SUBSTANCES INTO CELLS, AND USE OF SAID METHOD

(57) Abstract

A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell, is disclosed. Also different applications of said method are disclosed.

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A METHOD FOR INTRODUCING SUBSTANCES INTO CELLS, AND USE OF SAID METHOD

Field of the invention

The present invention relates to in vitro and in vivo methods for introducing substances into a mammalian stem cell and/or progenitor cells, as well as to use of such methods.

Background of the invention

For several years it has been clear that cellular mechanisms exist that allow cells to internalize nucleic acids. A new approach for chemotherapy has been developed based on the fact that addition of defined oligonucleotides (antisense inhibitors) to cells in tissue culture has been shown to block specific gene expression.

Previous studies have established that short single stranded DNAs are rapidly internalized by a variety of cultured cells (Bennett, R. M., Gabor, G. T. and Merritt, M. M., J. Clin. Invest. 76, 2182-2190 (1985); Loke, S. L., Stein, C. A., Zhang, X. H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J. S. and Neckers, L. M., Proc. Natl. Acad. Sci. USA 86, 3474-3478 (1989); Yakubov, L. A., Deeva, E. A., Zarytova, V. F., Ivanova, E. M., Ryte, A. S., Yurchenko, L. V., and Vlassov, V. V., Poc. Natl. Acad. Sci. USA 86, 6454-6458 (1989); Iversen, P. L., Zhu, S., Meyer, A., and Zon, G., Antisense Res. Dev. 2, 211-222 (1992); Wu-Pong, S., Weiss, T. L., and Hunt, C. A. Pharmacol. Res. 9, 1010-1017 (1992); Chan, T. M., Framton, G and Cameron, J. S., Clin. Exp. Immunol. 91, 110-114 (1993)). There are reports of DNA receptor structures that mediate uptake and destruction of DNA in human leucocytes ((Bennett, R. M., Gabor, G. T. and Merritt, M. M. J. Clin. Invest. 76, 2182-2190 (1985)).

However, naked DNA, RNA and oligonucleotides are in general unable to cross cellular membranes in vivo (Bo-

ado, R. J., Tsukamoto, H. and Pardridge, W. M. J. Pharm. Sci. 87, 1308-1315 (1998). Therefore, several strategies based on the binding of DNA to soluble carriers, receptor structures or conjugates (e.g. DNA binding proteins, conjugates of poly-L-lysine and an integrin receptor ligand) mediating the interaction with a transmembraneous transport system (see e.g. WO 96/15811; WO 94/25608; Kato, Y. and Sugiyama, Y. Crit. Rev. Ther. Drug Carrier syst. 14, 287-331 (1997)).

In general, replacement of neurons following degeneration or damage is not a characteristic of the mammalian brain. Neuronal loss is thus considered permanent. Prolonged postnatal neurogenesis has been described in the granule cell layer of the hippocampal formation (Altman, J. and Das, G. D., J. Comp. Neurol. 124: 319-335 (1965); Altman, J. and Das, G. D., Nature 214: 1098-1101 (1967); Caviness, V. S. jr., J. Comp Neurol. 151: 113-120 (1973); Gueneau, G., Privat, A., Drouet, J., and Court, L., Dev. Neurosci. 5, 345-358(1982); Eckenhoff, M. F. and Rakic, P., J. Neurosci. 8: 2729-2747(1988)). Cell genesis and neurogenesis have recently been shown to persist well into adulthood in man (Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A., Nordborg, C., Peterson, D. A., Gage, F. H., Nature Med. 4:1313-1317 (1998).

Newborn neurons in the granule cell layer express markers of differentiated neurons and have morphological characteristics corresponding to differentiated granulae cells (Kaplan, M. S. and Bell, D. H., J. Neurosci. 4: 1429-1441 (1984); Cameron, H. A., Woolley, C. S., McEwen, B. S., and Gould, E., Neuroscience 56: 337-344 (1993); Cameron, H. A., Woolley, C. S., and Gould, E., Brain Res. 611: 342-346 (1993)). Furthermore, they establish axonal processes into the mossy fiber pathway and form synaptic connections with their targets in hippocampus CA3 (Seki, T. and Arai, Y., J. Neurosci. 13: 2351-2358 (1993); Stanfield, B. B., and Trice, J. E., Exp. Brain Res. 72: 399-406 (1988)). The hippocampus is associated with spatial

learning and memory (McNamara, R. K., and Skelton, R. W., Brain Res. Rev. 18: 33-49 (1993)). The proliferation of progenitor cells can be influenced by the administration of N-methyl-D-aspartate (NMDA) receptor antagonists or by the removal of the adrenal glands (Cameron, H. A., and Gould, E., Neuroscience 61: 203-209 (1994); Cameron, H. A., Tanapat, P., and Gould, E., Neuroscience 82: 349-354 (1998)). Plasticity is reduced with increasing age, and recent studies have demonstrated that proliferation of progenitor cells also is decreased but not completely abolished with age (Kuhn, H., Dickinson-Anson, H., and Gage, F. H., J. Neurosci. 16: 2027-2033 (1996)). Stem cells, isolated through a time consuming and laborious tissue culture procedure, from the adult rodent brain has recently been transplanted into the brain of adult animals where they differentiate into cells with neuronal characteristics (Suhonen, J. O., Peterson, D. A., Ray, J., and Gage, F. H., Nature 383:624-627 (1996)). There are so far no known stem cell markers that are usable for rapid isolation of stem cells or progenitor cells from the adult central nervous system. This fact inhibits the therapeutic use of stem cells in humans. So far the detection of stem cells rely on indirect detection method using modified nucleotides that incorporates in to the genome in dividing cells during the S-phase of the cell cycle. Thereafter, the phenotype of the progeny can be detected using immunohistochemical methods. The limitation with this way of identifying progenitor progeny is that these cells no longer possess the stem cell or progenitor cell properties meaning that these cells lack the ability to self renew and to give rise to neurons, astroglia, or oligodendrocytes. Alternative strategies to isolate stemcells from rodents, based on either unselective dye staining, immunosorting with antibodies against the protein nestin expressed by all cells surrounding the ventricles, or unselective infection with viruses carrying the gene for a selectable marker, was recently pub-

lished (Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., Frisen, J., Cell 96: 25-34 (1999)). Neither of these methods is highly efficient and thus unsuitable for rapid isolation of stemcells from small human tissue samples. Therefore, it is of importance to identify a usable marker or property allowing for rapid isolation of stem or progenitor cells for therapeutic purposes e.g. autologous neural transplantation.

Summary of the invention

Due to the fact that mammalian progenitor cells and stem cells from the adult CNS lack specific marker molecules it has up to now been virtually impossible to perform rapid detection and isolation of those cells. During the work leading to the present invention it was found that progenitor cells and stem cells from the adult brain possess a highly efficient mechanism for uptake of nucleic acids, such as DNA. It was also found that it is possible to use said transport system in order to mark or tag progenitor cells and stem cells via administration of e.g. double stranded DNA either in linear form or in circular form (plasmids) which is taken up by the progenitor cells or the stem cells via direct interaction between the DNA and the cells, without the use of facilitating drugs, carriers, soluble receptors or chemicals or any special devices. The DNA is not immediately degraded. Instead, if the plasmid DNA contains the necessary components for expression, the aforementioned cells can be detected by the expression of plasmid cDNAs. If the DNA/plasmid, containing suitable elements for expression, cDNA and promotor, is incubated in the presence of progenitor cells or stem cells, said DNA is taken up efficiently and the protein corresponding to the cDNA is expressed by the progenitor cells or stem cells.

The invention is based on the use of this nucleic acid transport system in progenitor cells and stem cells

for different purposes. According to the present invention, it is possible to transfer DNA without the help or aid of e.g. viral vectors. The invention provides new methods to isolate progenitor cells and stem cells in vivo and in vitro. This isolation may be based on the expression from plasmid containing cDNA of a protein that enables selective identification and isolation based on immunoreactivity, or on the expression by DNA of a protein that enables selective identification and isolation based on the expression of fluorescent proteins, including FACS sorting. The invention also provides new methods to transport different substances with e.g. pharmaceutical effects into progenitor cells and/or stem cells.

The object of the invention is thus a method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane in vitro whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell. Said method may be performed both in vitro and in vivo.

The method is particularly suitable for isolation of progenitor cells or stem cells from the adult brain, for gene therapy, for cell sorting and for diagnostic procedures.

The characterizing features of the invention will be evident from the following description and the appended claims.

There are several advantages with the present invention compared with known strategies based on the binding of DNA to a soluble carrier, a receptor structure or a conjugate (such as a DNA binding protein, a conjugate of poly-L-lysine and an integrin receptor ligand) mediating the interaction with a transmembraneous transport system. One important advantage is that the invention does not rely on the binding of DNA to any soluble receptors or

carriers. Another important advantage is that it allows for the selective labeling of cells, due to the fact that only cells with the above described inherent transport mechanism are transfected.

Detailed description of the invention

The transport mechanism upon which the present invention is based and which is found in mammalian stem cells and progenitor cells from the brain, including human stem cells and progenitor cells from the brain, can be utilized in order to transport single or double stranded DNA or RNA into a cell and subsequently allow for the DNA or RNA to remain intact and undegraded in the cell. Cells in which this transport mechanism is found are especially adult derived neural stem cells and progenitor cells.

By utilizing this transport it is thus possible to insert nucleic acids into said cells.

These nucleic acids may either be used for their ability to make it possible to identify and thus isolate progenitor cells and stem cells from other cells, or for their pharmaceutical effects.

As stated above, the present invention relates to a method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said substance is brought into contact with said cell, whereby it is taken up by the cell via the inherent transport mechanism of the cell. The method may be used both in vitro and in vivo. The cells used in the method according to the invention are preferably derived from an adult.

The substance to be introduced into a cell according to the method is or comprises e.g. a single or double stranded, linear or circular DNA, or a single or double stranded RNA. The substance may also be a fusion molecule comprising a nucleic acid part and a protein part, or an expression vector containing a specific cDNA. The expres-

sion "expression vector" used herein relates to all vectors or plasmids consisting of a double stranded DNA structure comprising cDNA for a specific peptide or protein. Once this expression vector is taken up by the stem cells or progenitor cells it will lead to the synthesis of said peptide or protein.

When the substance is an expression vector it is preferably, according to one embodiment of the invention further commented on below, that the cDNA gives rise to a peptide or protein that activate proliferation and/or differentiation and/or lineage determination of said cells.

As stated above, the method according to the invention may be performed both in vitro, e.g. in a tissue or cell culture, and in vivo. When the method is performed in vivo, the cells into which the substance is transported are preferably cells in the central nervous system.

The methods according to the invention may be used for several different purposes, both diagnostic and therapeutic.

When the method is performed in vitro, it is especially suitable for the identification of progenitor cells and stem cells. When the methods according to the invention are used for the purpose of identification it is preferable that the substance that is to be introduced into said cells gives rise to a detectable signal or to a peptide or protein that enables selective identification of stem cells and progenitor cells. Said peptide or protein may then in its turn give rise to a detectable signal, as the case is for e.g. a fluorescent protein, or a marker protein. Examples of suitable markers for stem cells or progenitor cells are protein components of the transport system, such as receptors and carriers. The detectable signal may also be obtained by the use of tagged substances, such as a radioactively tagged nucleic acid.

It is especially interesting to be able to identify, and thereafter isolate, progenitor cells and stem cells in samples constituted of e.g. different structures of brain tissue taken out of a patient or cells cultured from a patient.

Once the stem cells or progenitor cells have been identified, they can be isolated from the other types of cells in the sample by any appropriate method known to man skilled in the art. The isolated cells can then for example be used in different tests, for diagnostic purposes or be propagated and transplanted to a patient.

The in vivo method can be used in order to identify, and subsequently isolate, cells in vivo, in a way similar to the in vitro method described above. When the method is performed in vivo, it is possible to identify, and thus isolate, stem cells and progenitor cells in different structures of the intact brain

It is also possible to propagate stem cells and progenitor cells with the methods according to the invention. This propagation can be performed both in vitro and in vivo. The cells, which optionally first may have been identified and isolated with the methods according to the invention, are then brought in contact with a substance that comprises or gives rise to peptide or protein that, once it is taken up by the cells, activate proliferation and/or differentiation and/or lineage determination of said cells.

It is also possible to use both the in vitro and the in vivo methods according to the invention for gene therapy. The substance that is brought into contact with the cells, and subsequently is transported into the cells, may then be a pharmaceutically active substance. It may also give rise to a pharmaceutically active substance once it is taken up by the cells. The substance may then e.g. be an expression vector comprising cDNA encoding the pharmaceutically active substance. The pharmaceutically active substance produced by the cDNA once it is taken up

by the cell may be a peptide or protein that will get transported out of the progenitor cell or stem cell to affect surrounding tissue or cells. Examples of such a peptide or protein are trophic factors, or other proteins exerting a desired action on neighboring cells and tissues. The peptide or protein produced by the cDNA may also be a substance that will either activate or inactivate proliferation, differentiation or specific lineage determination of the progenitor cells or stem cells either in order to be able to more easily isolate progenitors or stem cells or in order to induce the genesis of new neurons, astrocytes or oligodendrocytes from progenitors or stem cells in the brain or within progenitors or stem cells in a tissue culture for concomitant use for transplantation of said cells to patients. It is also possible to use a substance constituted of a fusion molecule between a nucleic acid, that enables the transport into the cells, and a pharmaceutically active protein.

When the gene therapy is performed in vivo, it can be used for treatment of neurological insult, disease, deficit or condition in a patient. The term "treatment" used herein relates to both treatment in order to cure or alleviate a disease or a condition, and to treatment in order to prevent the development of a disease or a condition. The treatment may either be performed in an acute or in a chronic way. The term "patient", as it is used herein, relates to any human or non-human mammal in need of treatment according to the invention.

It is possible to produce medicinal products for treatment of conditions due to disturbances of the normal function of stem cells or progenitor cells by attaching a pharmaceutically active compound to a nucleic acid. The nucleic acid will, when it is brought into contact with a stem cell or progenitor cell, be taken up into the cell by the inherent transport mechanism of the cell, and since the pharmaceutically active compound is attached to the nucleic acid it too will be transported in to the

cell. Such medicinal products may also comprise other substances, such as an inert vehicle, or pharmaceutical acceptable adjuvants, carriers, preservatives etc., which are well known to persons skilled in the art. It is preferable that such medicinal products are administered to a patient by infusion into the cerebral ventricles through a surgically inserted canula or via a syringe inserted between lumbar vertebras and into the spinal fluid.

The methods according to the invention can also be used in order to test or screen a protein or a detectable signal. In a screening or test application the invention is used in with stemcells that take up DNA including cDNA coding for a protein of interest that are subject to screening or testing. Examples of proteins are receptors that can be used for screening new receptor agonists. The transport and uptake of and subsequent expression from plasmids in cells according to the invention can be used in detector devices and screening devices where expression of specific proteins like receptors or enzymes are desired. The advantage of the present invention compared with conventional transfection techniques in which drugs or compounds that facilitate DNA uptake are necessary for efficient uptake and expression, is the high efficiency and lack of need for drugs, compounds or chemicals to facilitate uptake and subsequent expression of proteins.

The invention will now be further explained in the following example. This example is only intended to illustrate the invention and should in no way be considered to limit the scope of the invention.

Brief description of the drawing

In the example below, reference will be made to the accompanying figure, wherein:

Figure 1A is a fluorescence photomicrograph showing the result of incubation of progenitor cells in medium with 50 µg/ml of a plasmid containing the cDNA for GFP;

Figure 1B a lightmicroscopic image showing the same result as figure 1A;

Figure 1C is a fluorescence photomicrograph showing the result of incubation of progenitor cells in medium with 50 µg/ml of another plasmid not containing the GFP gene; and

Figure 1D a lightmicroscopic image showing the same result as figure 1B.

Example

Expression by progenitor cells from mature rat brain of a fluorescent marker

The expression of green fluorescent protein (GFP) was examined in progenitor cells isolated from the adult hippocampus. Progenitor cells (Palmer, T. D., Ray, J. and Gage, F. H. (1995) Mol. Cell. Neurosci. 6: 474-486.) and cos-7 cells were cultured according to standard procedures and plated onto 1-inch circular coverslips coated with poly-D-ornithine and lamilin. The cells were incubated with plasmids containing the cDNA for GFP, and plasmids deficient of the GFP gene, respectively, in a humid atmosphere at 37°C with 5% CO_2 and 95% air for 10 minutes. The cells were cultured for 48 h, following DNA exposition.

Thereafter the expression of the fluorescent protein was detected using an inverted Leica DMIRB microscope equipped for fluorescence microscopy. The cells were viewed in the microscope using excitation of GFP at 488 nm using an Ar-ion laser (Spectra Physics model 2025-05, Sunnyvale, CA). The laser light was sent through a 488-line interference filter followed by a spinning disk to break the coherence and scatter the laser light. The laser was collected by a lens and sent through a fluorescein filter cube (Leica I-3) into the objective to excite the fluorophores. The resulting fluorescence was collected by the same objective and the image was detected by a 3-chip color CCD-camera (Panasonic) and recorded at

25 Hz frame collection rate by a Super VHS (Panasonic SVHS AG-5700). The CCD images were digitized from tape and processed for presentation.

When progenitor cells were incubated in medium with 50 µg/ml of a plasmid containing the cDNA for GFP for 10 minutes without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48h before detection, they were highly fluorescent. Figure 1A is a fluorescence photomicrograph showing this result, and Figure 1B shows the respective lightmicroscopic image.

In contrast, when progenitor cells from adult rat brain were incubated with other plasmids not containing the GFP gene, no fluorescence was observed. Figure 1C and 1D show the respective images when GFP deficient plasmid DNA were used.

Also, when progenitor cells were incubated with plasmid containing the gene expressing b-galactosidase 50 µg/ml, without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48 h before detection, cells expressed b-galactosidase activity.

It was also found that kidney-derived Cos-7 cells that were incubated in medium with 50 µg/ml of a plasmid containing the cDNA for GFP for 10 minutes without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48h before detection, lack expression of green fluorescent protein (GFP). Detection and experimental procedures for this experiment was identical to that for progenitor cells exposed to plasmid containing the cDNA for GFP.

Also, when cos-7 cells were incubated with plasmid containing the gene expressing b-galactosidase, without addition of chemicals that facilitate uptake or transport of DNA, and thereafter grown for 48 h before detection, they displayed a lack of expression of b-galactosidase activity.

It is clear from the above experiments that progenitor cells from adult rat brain has a capacity to in vitro transport double-stranded DNA plasmids to their interiors, and to synthesize the proteins that the DNA sequence codes for.

CLAIMS

1. A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane in vitro whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell.

2. A method according to claim 1, wherein said cell is derived from an adult.

3. A method according to claim 2, wherein said method is performed in a humid atmosphere at 37°C.

4. A method according to any one of the claims 1-3, wherein said substance is or comprises a single or double stranded, linear or circular DNA.

5. A method according to any one of the claims 1-3, wherein said substance is or comprises a single or double stranded RNA.

6. A method according to any one of the claims 1-3, wherein said substance is a fusion molecule comprising a nucleic acid part and a protein part.

7. A method according to any one of the claims 1-3, wherein said substance is an expression vector containing a specific cDNA.

8. A method according to claim 7, wherein said cDNA gives rise to a peptide or protein that activate proliferation and/or differentiation and/or lineage determination of said cells.

9. A method according to any one of the claims 1-6, wherein said substance gives rise to a detectable signal.

10. A method according to claim 7, wherein said cDNA gives rise to a peptide or protein that enables selective identification of stem cells and/or progenitor cells.

11. A method according to claim 10, wherein said peptide or protein gives rise to a detectable signal.

12. A method according to claim 11, wherein said protein is a fluorescent protein.

13. A method according to claim 11 or 12, wherein said detectable signal is due to a radioactively tagged nucleic acid.

14. A method according to any one of the claims 1-13, wherein said cell is a cell in a tissue or cell culture.

15. Use of a method according to any one of the claims 1-14, for identification of progenitor cells and/or stem cells.

16. Use according to claim 15, wherein said cells after identification is isolated from surrounding cells of other types.

17. Use of a method according to any one of the claims 1-14, for gene therapy.

18. Use of a method according to claim 6 and 17, wherein said protein part consists of a pharmaceutically active protein.

19. Use of a method according to claim 8, for propagation of neural cells.

20. Use according to claim 18, wherein said propagated neural cells are suitable for transplantation to patients.

21. Use of a method according to any one of the claims 1-14, for detection of a medicinal product comprising cDNA containing expression plasmids.

22. Use of a method according to any one of the claims 1-14, for diagnostic purposes.

23. Use of a method according to any one of the claims 8-13, wherein said protein or detectable signal allows for testing or screening of aforementioned protein or signal.

24. A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or

a component within said cell membrane in vivo, whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell.

25. A method according to claim 24, wherein said cell is derived from an adult.

26. A method according to claim 24, wherein said substance is or comprises a single or double stranded, linear or circular DNA.

27. A method according to claim 24, wherein said substance is or comprises a single or double stranded RNA.

28. A method according to claim 24, wherein said substance is a fusion molecule comprising a nucleic acid part and a protein part.

29. A method according to claim 24, wherein said substance is an expression vector containing a specific cDNA.

30. A method according to claim 29, wherein said cDNA gives rise to a peptide or protein that activate proliferation and/or differentiation and/or lineage determination of said cells.

31. A method according to claim 24, wherein said substance gives rise to a detectable signal.

32. A method according to claim 29, wherein said cDNA gives rise to a peptide or protein that enables selective identification of stem cells and/or progenitor cells.

33. A method according to claim 32, wherein said peptide or protein gives rise to a detectable signal.

34. A method according to claim 33, wherein said protein is a fluorescent protein.

35. A method according to claim 33, wherein said detectable signal is due to a radioactively tagged nucleic acid.

36. A method according to any one of the claims 1-13, wherein said cell is a cell in the central nervous system of a patient.

37. Use of a method according to claim 24, for identification of progenitor cells and/or stem cells.

38. Use according to claim 37, wherein said cells after identification is isolated from surrounding cells of other types.

39. Use of a method according to any one of the claims 24-38 for gene therapy.

40. Use of a method according to claim 28, wherein said protein part consists of a pharmaceutically active protein.

41. Use of a method according to claim 30, for propagation of neural cells.

42. Use of a method according to any one of the claims 24-36, for detection of a medicinal product comprising cDNA containing expression plasmids.

43. Use of a method according to any one of the claims 24-36, for diagnostic purposes.

44. Use of a method according to any one of the claims 30-35, wherein said protein or detectable signal allows for testing or screening of aforementioned protein or signal.

45. Use of a method according to claim 24, for treatment of neurological insult, disease, deficit or condition.

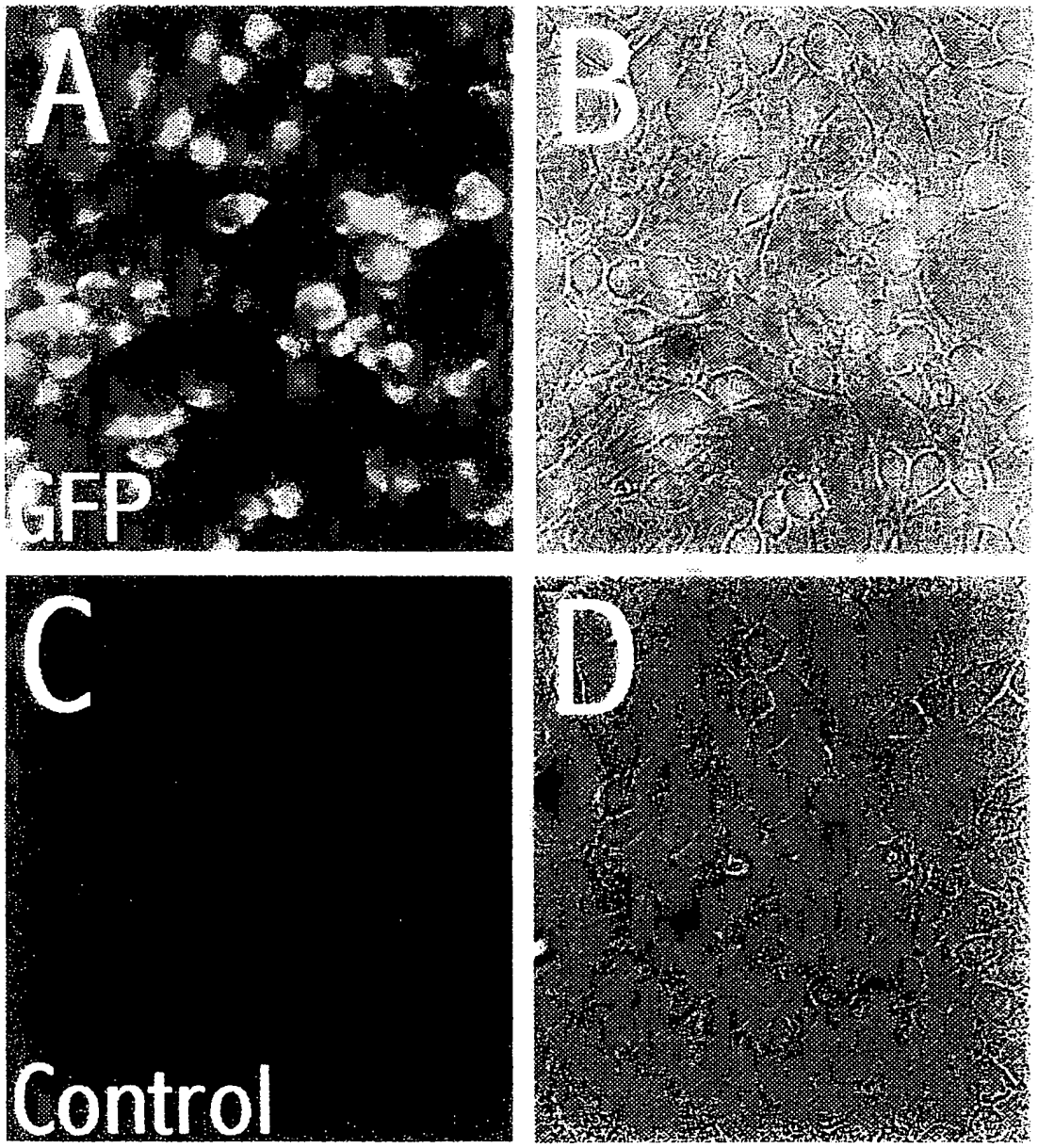

Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/00073

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 15/64, A61K 48/00, C12Q 1/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9615811 A1 (IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE), 30 May 1996 (30.05.96), page 3, line 32 - page 5, line 30; page 6, line 32 - page 7, line 2, example 2 --	1-45
A	WO 9425608 A1 (BAYLOR COLLEGE OF MEDICINE), 10 November 1994 (10.11.94), page 13, column 15 - page 14, line 20, examples 11,12 --	1-45
A	Journal of Pharmaceutical Sciences, Volume 87, No 11, November 1998, Ruben J. Boado et al, "Drug Delivery of Antisense Molecules to the Brain for Treatment of Alzheimer's Disease and Cerebral AIDS" page 1308 - 1315 --	1-45

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2000	16 -05- 2000
Name and mailing address of the ISA Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Hampus Rystedt/LR Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/00073

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Critical Reviews in the Therapeutic Carrier Systems, Volume 14, No 3, 1997, Yukio Kato et al, "Targeted Delivery of Peptides, Proteins and Genes by Receptor-Mediated Endocytosis" page 287 - 331 --	1-45
A	US 5750376 A (SAMUEL WEISS ET AL), 12 May 1998 (12.05.98), column 20, line 58 - column 21, line 15 --	1-45
A	WO 9744442 A1 (NEURALSTEM BIOPHARMACEUTICALS), 27 November 1997 (27.11.97) -- --------	1-45

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/00073

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 39,40,43,45
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 39, 40, 43 and 45 relate to therapeutic or diagnostic methods, a search has been carried out and been based on the alleged effects of the claimed compounds/methods.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/99

International application No.
PCT/SE 00/00073

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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INTERNATIONAL SEARCH REPORT
Information on patent family members

02/12/99

International application No.
PCT/SE 00/00073

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/99

International application No.
PCT/SE 00/00073

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5750376 A	12/05/98	FI 953569 A	25/09/95
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		US 5753506 A	19/05/98

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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C12N 15/64, A61K 48/00, C12Q 1/68

A1

(11) International Publication Number: WO 00/42202

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(71) Applicant *(for all designated States except US)*: A+ SCIENCE INVEST AB [SE/SE]; Box 3096, S-400 10 Göteborg (SE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ERIKSSON, Peter [SE/SE]; Dr Saléns gata 10, S-413 22 Göteborg (SE). ORWAR, Owe [SE/SE]; Vasagatan 35, S-411 37 Göteborg (SE).

(74) Agent: AWAPATENT AB; Box 11394, S-404 28 Göteborg (SE).

(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (Utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
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Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD FOR INTRODUCING SUBSTANCES INTO CELLS, AND USE OF SAID METHOD

(57) Abstract

A method for introducing a substance comprising a nucleic acid into a mammalian neural stem cell or progenitor cell, characterized in that said nucleic acid directly interacts with the cell membrane of said cell or a component within said cell membrane whereby the substance comprising said nucleic acid is taken up by the cell via the inherent transport mechanism of the cell, is disclosed. Also different applications of said method are disclosed.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/00073

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 15/64, A61K 48/00, C12Q 1/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9615811 A1 (IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE), 30 May 1996 (30.05.96), page 3, line 32 - page 5, line 30; page 6, line 32 - page 7, line 2, example 2 --	1-45
A	WO 9425608 A1 (BAYLOR COLLEGE OF MEDICINE), 10 November 1994 (10.11.94), page 13, column 15 - page 14, line 20, examples 11,12 --	1-45
A	Journal of Pharmaceutical Sciences, Volume 87, No 11, November 1998, Ruben J. Boado et al, "Drug Delivery of Antisense Molecules to the Brain for Treatment of Alzheimer's Disease and Cerebral AIDS" page 1308 - 1315 --	1-45

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
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13 April 2000	16 -05- 2000
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INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Critical Reviews in the Therapeutic Carrier Systems, Volume 14, No 3, 1997, Yukio Kato et al, "Targeted Delivery of Peptides, Proteins and Genes by Receptor-Mediated Endocytosis" page 287 - 331 --	1-45
A	US 5750376 A (SAMUEL WEISS ET AL), 12 May 1998 (12.05.98), column 20, line 58 - column 21, line 15 --	1-45
A	WO 9744442 A1 (NEURALSTEM BIOPHARMACEUTICALS), 27 November 1997 (27.11.97) --	1-45

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 00/00073

Box I Observations where certain claims were found unsearchable (Continuation f item 1 of first sheet)

This international search report has not been established in respect f certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 39,40,43,45
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 39, 40, 43 and 45 relate to therapeutic or diagnostic methods, a search has been carried out and been based on the alleged effects of the claimed compounds/methods.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/99

International application No.
PCT/SE 00/00073

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